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REVIEW

Sox9 Function in Craniofacial Development and Disease

Young-Hoon Lee[1,2] **and Jean-Pierre Saint-Jeannet**[2]*

[1]Department of Oral Anatomy, School of Dentistry and Institute of Oral Biosciences, Chonbuk National University, Jeonju, South Korea

[2]Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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Summary: The Sox family of transcriptional regulators has been implicated in the control of a broad array of developmental processes. One member of this family *SOX9* was first identified as a candidate gene for campomelic dysplasia (CD), a human syndrome affecting skeletal, and testis development. In these patients most endochondral bones of the face fail to develop resulting in multiple defects such as micrognathia, cleft palate, and facial dysmorphia. In this review we describe *Sox9* expression during embryonic development and summarize loss of function experiments in frog, fish, and mouse embryos highlighting the role of *Sox9* in regulating morphogenesis of the face. We also discuss the mutations in and around *SOX9* responsible for craniofacial defects in CD patients. genesis 49:200–208, 2011. **© 2011 Wiley-Liss, Inc.**

Key words: craniofacial; cartilage; neural crest; Sox9; campomelic dysplasia

INTRODUCTION

The *Sox* (*S*ry HMG-b*ox*) gene family of transcriptional regulators was discovered in the early 1990s through the cloning of *Sry*, the mammalian testis-determining gene (Lovell-Badge, 2010). *Sox* genes are part of a larger family of high-mobility group (HMG) proteins, defined by their electrophoretic mobility on SDS-PAGE. Sox proteins bind DNA by means of their HMG domain, allowing them to function as transcription factors. This domain is highly conserved among Sox factors, which typically recognize a similar motif on the DNA: (A/T)(A/T)CAA(A/T)G. All Sox proteins bind DNA with low affinity, and are thought to require cofactors to stabilize their interactions with the DNA and mediate their transcriptional activity (Kamachi *et al*., 2000; Wilson and Koopman, 2002). Because most Sox factors are expressed in more than one cell lineage, cell type-specific cofactors are also required to confer specificity for various promoters (Kamachi *et al*., 2000; Wilson and Koopman, 2002). According to their sequence homologies within and outside the HMG domain, Sox proteins have been sorted into 10 groups, A through J, with Sry being allocated to the SoxA group (Bowles *et al*., 2000; Schepers *et al*., 2002).

Sox9 belongs to the SoxE group, which also comprises Sox8 and *Sox10*. SoxE proteins are critical for the development of neural crest progenitors (Haldin and LaBonne, 2010; Hong and Saint-Jeannet, 2005). In all vertebrates one or more SoxE proteins are implicated in the specification, multipotency, and survival of neural crest cells. Later in embryogenesis, individual SoxE proteins direct the development of distinct neural crest derivatives, including melanocytes, chondrocytes, and sensory neurons (Haldin and LaBonne, 2010; Hong and Saint-Jeannet, 2005). SoxE factors are also essential for many aspects of peripheral and central nervous system development where they play a major role in oligodendrocyte and Schwann cell formation (Stolt and Wegner, 2010; Wegner, 2008). *SOX9* was first identified as a candidate gene for campomelic dysplasia (CD), a condition characterized by skeletal defects, cranial dismorphology and sex reversal (Foster *et al*., 1994; Wagner *et al*., 1994). *Sox9* turns out to be a master regulator of carti-

*Correspondence to: Jean-Pierre Saint-Jeannet, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104, USA. E-mail: saintj@vet.upenn.edu

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lage development. It has essential roles in the specification and differentiation of mesenchymal cells toward the chondrogenic lineage in all developing skeletal elements. Sox9 binds to and controls the activity of chondrocyte-specific enhancers of several genes critical for chondrocyte differentiation, including Type-II collagen, *Col2a1*, the major matrix protein of the mature cartilage (Lefebvre and de Combrugghe, 1998; Lefebvre and Smits, 2005).

Here we summarize the expression pattern of *Sox9* as it relates to craniofacial development and review some of the key features of *Sox9* function during morphogenesis of the face. We also discuss the mutations in or near *SOX9* causative of the craniofacial defects in CD patients.

Developmental Expression of *Sox9*

During embryonic development in all species examined *Sox9* is expressed in a broad array of tissues including the gonad, otic vesicle, lung, notochord, neural tube, pancreas, and cardiac cushions (Chiang *et al*., 2001; Ng *et al*., 1997; Spokony *et al*., 2002; Wright *et al*., 1995; Zhao *et al*., 1997). The multi-lineage expression of *Sox9* suggests the existence of complex regulatory mechanisms to control its tissue-specific expression during embryogenesis (Bagheri-Fam *et al*., 2006). More relevant to the focus of this review, *Sox9* is also detected in neural crest progenitors at the lateral edge of the neural plate and is maintained in cranial neural crest cells as they populate the pharyngeal arches in the head region (Hong and Saint-Jeannet, 2005).

The neural crest is a multipotent population of migratory cells unique to the vertebrate embryo with the remarkable ability to give rise to a broad range of derivatives. The premigratory neural crest can be divided into five distinct and partially overlapping domains along the antero-posterior axis, the cranial, cardiac, vagal, trunk, and sacral neural crest, each forming a specific set of derivatives in the periphery (LeDouarin and Kalcheim, 1999). Cranial neural crest cells delaminate from the posterior midbrain and individual rhombomeres in the hindbrain, and migrate into the pharyngeal arches to give rise to skeletal elements of the face, and contribute to the pharyngeal glands (thymus, thyroid, and parathyroid) and portions of the cardiovascular system (Fig. 1; LeDouarin and Kalcheim, 1999). In each pharyngeal arch the neural crest will contribute to a specific set of skeletal elements. The first pharyngeal arch has two components, the maxillary prominence forming the premaxilla, maxilla, zygomatic bone, and temporal bone; and the mandibular prominence forming the middle ear bones, malleus and incus, and Meckel's cartilage. The second pharyngeal arch gives rise to the stapes, styloid process and lesser horn of the hyoid, while the greater horn of the hyoid is derived from the third pharyngeal arch. The elements derived from the most caudal pharyngeal arches (4–6) form the laryngeal cartilages (Fig. 1; Minoux and Rijli, 2010).

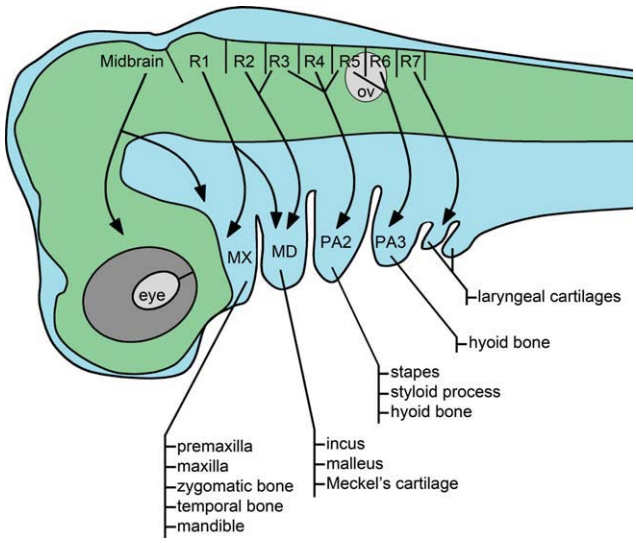

FIG. 1. Pattern of cranial neural cell migration and their skeletal derivatives. In the mammalian embryo neural crest cells delaminate from the posterior midbrain and individual rhombomeres (R1–R7) in the hindbrain, and migrate into the pharyngeal arches (PA). Neural crest cells migrate in a stereotypical pattern based on their origin in the hindbrain (arrows). In each arch the neural crest contributes a specific set of skeletal elements as indicated. The first pharyngeal arch has two parts the maxillary (MX) and mandibular (MD) prominences. The most caudal pharyngeal arches form laryngeal cartilages. Lateral view, anterior to left, dorsal to top. ov, otic vesicle.

In zebrafish two orthologs of *Sox9* have been identified: *sox9a* and *sox9b* (Chiang *et al*., 2001). *sox9b* is expressed earlier than *sox9a* in progenitors of the neural crest (Li *et al*., 2002; Yan *et al*., 2002, 2005). Both genes are co-expressed in the premigratory trunk and cranial neural crest, though *sox9b* is expressed at higher levels than *sox9a*. *sox9b* expression is downregulated in neural crest cells shortly after they initiate their migration toward the pharyngeal arches (Li *et al*., 2002; Yan *et al*., 2005). While *sox9b* expression is reactivated once neural crest cells reach the pharyngeal arches, *sox9a* is expressed at higher levels than *sox9b* in these structures (Yan *et al*., 2005). Early on *sox9a* is detected in the pre-condensed mesenchyme of the pharyngeal arch primordial, whereas *sox9b* expression in the mesenchyme of the pharyngeal arch starts just before hatching (Chiang *et al*., 2001; Yan *et al*., 2002). The pectoral fin rudiments (a cranial neural crest derivative) also express *sox9b* (Chiang *et al*., 2001). Craniofacial cartilages begin to condensate and differentiate between day 2 and 3. *sox9a* and *Sox9b* expression persists during chondrogenesis in essentially all the elements derived from the pharyngeal arches and in the neurocranium (Chiang *et al*., 2001).

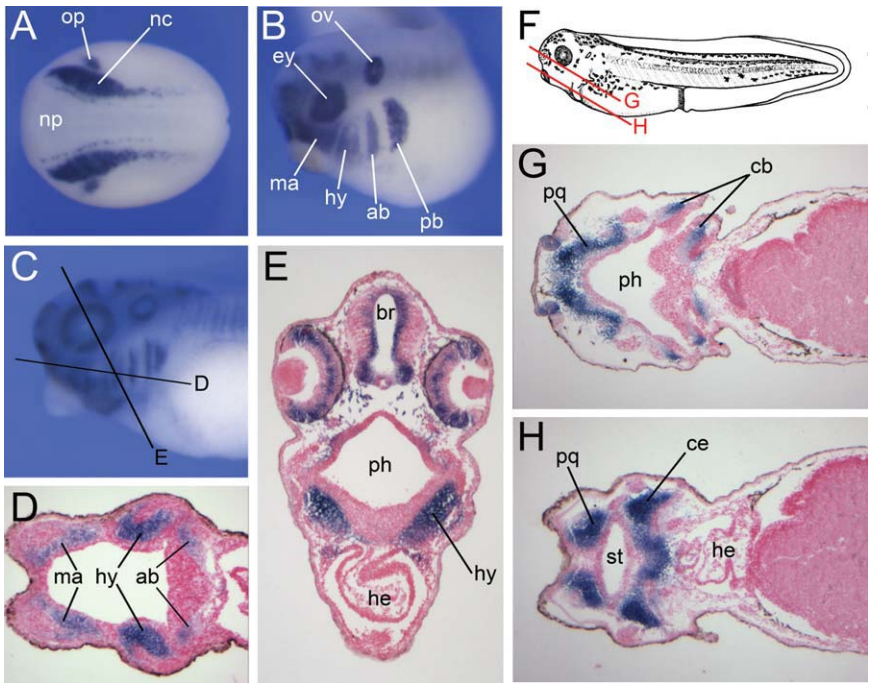

FIG. 2. Developmental expression of Xenopus *Sox9* in the neural crest lineage. (**A**) By in situ hybridization, at the end of gastrulation *Sox9* is detected in neural crest progenitors (nc) at the lateral edge of the neural plate (np), and in the presumptive otic placode (op). Dorsal view, anterior to left. (**B**) At the tailbud stage *Sox9* is detected in the four streams of cranial neural crest migrating toward the pharyngeal arches, the mandibular (ma), hyoid (hy), anterior branchial (ab), and posterior branchial (pb) neural crest. Other domains of expression include the developing eye (ey) and the otic vesicle (ov). Lateral view, anterior to left, dorsal to top. (**C**) *Sox9* expression in the head region of a Stage 35 embryo (Nieuwkoop and Faber, 1967). Lateral view, anterior to left, dorsal to top. The black lines indicate the level of the sections shown in the subsequent panels. (**D,E**) Sections showing *Sox9* expression in the mesenchyme of the pharyngeal arches. (**F**) Diagram of a Stage 40 embryo, after Nieuwkoop and Faber (1967). Lateral view, anterior to left, dorsal to top. The red lines indicate the level of the sections shown in the subsequent panels. (**G,H**) *Sox9* is detected in all differentiating cartilage elements, including the palatoquadrate (pq), ceratobranchial (cb), ceratohyal (ce), and Meckel's cartilage (not shown). br, brain; he, heart; ph, pharynx; st, future stomodeum.

In the frog, *Xenopus laevis*, *Sox9* is also first detected in the neural crest forming regions at the end of gastrulation (Fig. 2; Spokony *et al.*, 2002). As the neural tube closes, *Sox9* expression persists in both the cranial and trunk neural crest. At the early tailbud stage as neural crest cells start to migrate in the cranial region, *Sox9* is strongly expressed in all four streams of migrating cranial neural crest, the mandibular, hyoid, anterior and posterior branchial neural crest, as well as at the dorsal midline in the trunk neural crest (Fig. 2; Spokony *et al.*, 2002). *Sox9* expression is subsequently downregulated in the trunk as cranial neural crest cells accumulate in the pharyngeal arches. *Sox9* expression persists in the mesenchyme of the pharyngeal arch primordia until the swimming tadpole stage (Fig. 2; Kerney *et al.*, 2007; Spokony *et al.*, 2002). Later in development *Sox9* is mostly restricted to the condensing mesenchyme corresponding to the future palatoquadrate, ceratohyal, ceratobranchial, and Meckel's cartilages (Fig. 2; Gross and Hanken, 2008; Kerney *et al.*, 2007).

Sox9 expression in the mouse neural crest is first detected around E8.5 at the dorsal tip of the closing neural tube in the hindbrain region, as well as in the neural folds in the trunk region (Ng *et al.*, 1997; Zhao *et al.*, 1997). In the mouse, neural crest cells start to migrate before the neural tube is fully closed, and *Sox9* is detected in cranial neural crest cells as they migrate away from the neuroepithelium. At E9.5, *Sox9* is expressed in the mesenchyme of the frontal nasal mass and in the first, second and third pharyngeal arches (Ng *et al.*, 1997; Wright *et al.*, 1995; Zhao *et al.*, 1997). *Sox9* expression is subsequently associated with prechondrogenic mesenchyme condensed into cartilage primordia throughout the developing skeleton. In E14.5 embryos, in which most skeletal structures have formed, *Sox9* is abundantly expressed at the base of the skull, in cartilage precursors of the supraoccipital bones, nasal, hyoid and Meckel's cartilages (Ng *et al.*, 1997; Wright *et al.*, 1995; Zhao *et al.*, 1997).

It is important to mention that in all species examined, once cranial neural crest cells have settled in the pharyngeal arches, *Sox9* is co-expressed with *Col2a1* in the pharyngeal arch mesenchyme (Chiang *et al.*, 2001; Kerney *et al.*, 2007, Ng *et al.*, 1997; Zhao *et al.*, 1997), consistent with the predicted function of *Sox9* in the regulation of chondrocyte development.

Sox9 Function in Craniofacial Development

As discussed above, the expression of *Sox9* in the developing neural crest and pharyngeal arch mesenchyme has been well conserved during evolution suggesting a conserved function for *Sox9* in craniofacial development across species. Loss-of-function experiments have been especially critical to understand the specific requirement for *Sox9* in the morphogenesis of the face. Here we summarize some of these findings in frog, fish, and mouse embryos.

In Xenopus embryos, *Sox9* knockdown by means of morpholino antisense oligonucleotides resulted in embryos exhibiting a specific loss of early neural crest markers, including *Snail2*, *FoxD3*, and *Sox10* (Aoki *et al*., 2003; Lee *et al*., 2004; Spokony *et al*., 2002). Later in development, the loss of neural crest progenitors correlated with a number of defects in skeletal elements derived from the cranial neural crest while the development of melanocytes, a trunk neural crest derivative, was largely unaffected. Craniofacial defects included a complete loss of Meckel's cartilage and defective ceratohyal and ceratobranchial cartilages (Spokony *et al*., 2002), elements derived from the mandibular, hyoid, and branchial neural crest, respectively (Gross and Hanken, 2008; Sadaghiani and Thiebaud, 1987). The basihyal, a mesoderm-derived cartilage that forms at the midline (Gross and Hanken, 2008; Sadaghiani and Thiebaud, 1987), was unaffected in these embryos (Spokony *et al*., 2002).

These defects were interpreted as the result of a specific depletion of the cranial neural crest progenitor pool, consistent with a role of *Sox9* in neural crest specification. Yet, it is likely that *Sox9* may also have a later role in the differentiation of neural crest cells once they reach the pharyngeal arches, a possibility that could not be evaluated with this type of knockdown approach. In another set of experiments embryos were injected with an inducible inhibitory mutant of *Sox9* activated at the neurula stage to bypass the early requirement for *Sox9* in neural crest specification. In these embryos the pattern of cranial neural crest migration was unperturbed suggesting that *Sox9* is dispensable for neural crest cells migration in the frog (Lee *et al*., 2004). However the craniofacial phenotype of these embryos has not been analyzed.

The situation is a little different in zebrafish due to the presence of two *sox9* orthologs, which carry distinct but complementary functions. In the *sox9a* zebrafish mutants, *jellyfish*, neural crest specification and migration occur normally, however these embryos have reduced expression of *col2a1* and lack all cranial cartilage, with the exception of a few cells of the ceratohyal (Yan *et al*., 2002). *sox9a* is not required for neural crest specification or pharyngeal cartilage condensation, but appears to play a later role in the subsequent differentiation of these cells by regulating the stacking of chondrocytes and the shaping of individual cartilages (Yan *et al*., 2002). The analysis of the phenotype of *sox9b* morpholino knockdown and mutants revealed distinct roles for *sox9a* and *sox9b* in neural crest and cartilage development (Yan *et al*., 2005). *sox9b* mutant have reduced first and second pharyngeal arch cartilages. In *sox9b* homozygous mutants chondrocytes stack normally but are reduced in number, suggesting a specific role of *sox9b* is regulating survival of these precursors (Yan *et al*., 2005). Each single mutant retains different amounts of pharyngeal cartilages, the double mutants are completely lacking cranial cartilages (Yan *et al*., 2005).

sox9b is expressed earlier than *sox9a* in neural crest progenitors and appears to carry a function in neural crest specification similar to the one described for Xenopus *Sox9* (Spokony *et al*., 2002). *sox9b* mutant embryos show reduced expression of the early neural crest markers *snail1b*, *foxd3*, and *sox10* at the neural plate border. Moreover, in overexpression studies, *sox9a* and *sox9b* have a positive regulatory influence on each other's expression and cause ectopic expression of several early neural crest markers consistent with an early function of *sox9b* in neural crest specification (Yan *et al*., 2005).

Sox9 homozygous mutant mouse embryos die midway through gestation, too early to evaluate any cranial malformation (Bi *et al*., 1999). *Sox9* heterozygous mutants die after birth with major skeletal defects, characterized by hypoplasia of all bones formed by endochondral ossification of cartilage, including a shortened jaw and cleft palate reminiscent of the malformations seen in CD patients (Bi *et al*., 2001). The skeleton of the body forms either by endochondral or intramembranous bone formation. Endochondral bone formation is the process by which bone develops from cartilage, while in intramembranous ossification mesenchymal cells differentiate directly into osteoblasts. The neural crest-derived craniofacial skeleton develops using both mechanisms.

To evaluate the role of *Sox9* in the neural crest lineage, *Sox9* was inactivated in neural crest cells by means of a Wnt1-Cre transgene (Mori-Akiyama *et al*., 2003). Sox9;Wnt1-Cre embryos died at birth from respiratory distress because of a large cleft palate. Newborn mutants exhibited craniofacial deformities characterized by a domed skull, a short snout, and short mandibles. Skeletal preparations of mutant skulls showed that all of the cartilages and endochondral bones including the basisphenoid and presphenoid were missing. Among the first pharyngeal arch-derived skeletal elements of the mutants, all intramembranous bones were conserved. In contrast, the endochondral bones, such as malleus and incus, and the nasal capsule were totally absent. Meckel's cartilage was also completely absent,

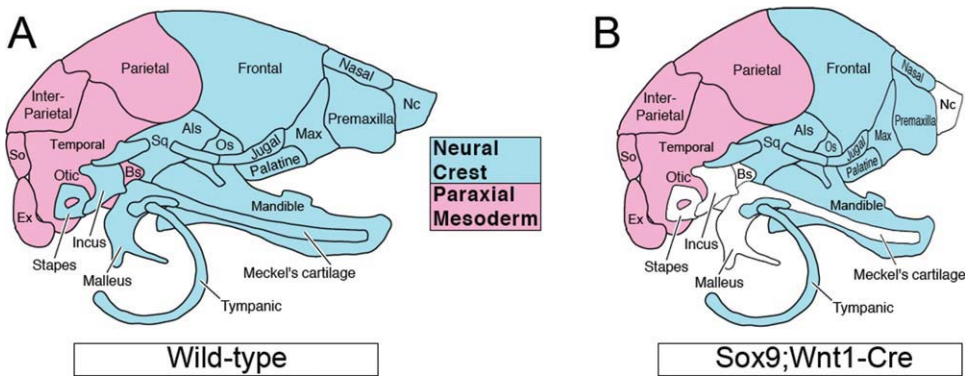

FIG. 3. Diagram illustrating the craniofacial defects observed in Sox9;Wnt1-Cre mouse embryos. (**A**) Diagram showing the paraxial mesoderm (red) and neural crest (blue) contribution to the mouse head skeleton. Lateral view, anterior to right. Als, Alisphenoid; Bs, Basisphenoid; Ex, Exooccipital; Nc, Nasal capsule; Os, Orbitosphenoid; So, Supraoccipital; Sq, Squamosal (modified from Noden and Schneider, 2006). (**B**) Sox9;Wnt1-Cre mouse embryos have a domed skull, a short snout and short mandibles. In these animals the missing skeletal elements of neural crest origin are depicted in white.

and the mandibles were smaller than those of the wild type embryos. The skeletal elements derived from the second and third branchial arches, such as the stapes, the hyoid bone and the styloid process were missing in the mutant embryos (see Fig. 3). These defects result not from abnormal migration or increased apoptosis in the cranial neural crest compartment, but rather from an inability of the post-migrating cranial neural crest cells to differentiate into chondrocytes (Mori-Akiyama *et al*., 2003). Early on, the expression of *Foxd3*, *Snail*, and *Sox10* was unaffected in the premigratory neural crest of mutant embryos suggesting that neural crest specification is initiated normally in the absence of *Sox9* (Cheung *et al*., 2005), unlike what has been reported in zebrafish and Xenopus (Spokony *et al*., 2002; Yan *et al*., 2005). This difference in the early requirement for *Sox9* in neural crest specification may reflect species-specific differences in the relative importance of the three SoxE proteins during development of the neural crest lineage (Haldin and LaBonne, 2010; Hong and Saint-Jeannet, 2005).

Cleft palate is a major defect observed in *Sox9* mutant mouse embryos (Bi *et al*., 2001; Mori-Akiyama *et al*., 2003). In mutant animals the development of the palate is arrested after the initial outgrowth of the palatal shelves. *Sox9* is normally expressed within the subepithelial mesenchyme of the palatal shelves before and during fusion, and therefore is likely to be required for the growth and fusion process of the palatal shelves (Nie, 2006; Yamashiro *et al*., 2004). *Sox9* mutants also show delayed posterior frontal suture closure. The posterior frontal suture is a mesenchymal structure derived from the cranial neural crest, and suture closure is a process occurring through endochondral ossification. In wild-type embryos, *Sox9* expression is typically upregulated within the suture mesenchyme prior to closure (Sahar *et al*., 2005).

In summary, these studies show that during craniofacial development *Sox9* is required in neural crest cells that will generate both the chondrocyte and osteoblast lineages participating in endochondral bone formation. However, *Sox9* is dispensable for the osteoblast lineage in intramembranous skeletal elements derived from the neural crest. Additionally, in fish and frogs, *Sox9* appears to have also an early function in neural crest specification.

Sox9 Mutations and the Craniofacial Features of CD Patients

Campomelic dysplasia (CD; OMIM # 114290) is a rare autosomal dominant skeletal dysmorphology syndrome characterized by congenital bowing of the long bones (campomelia), hypoplastic scapulae, 11 pairs of ribs, pelvic and spinal malformations, clubbed feet, facial dysmorphia and Pierre Robin sequence, which includes micrognathia (small lower jaw), macroglossia (enlarged tongue), and cleft palate (Mansour *et al*., 1995; Maroteaux *et al*., 1971). Campomelia is absent in about 10% of the cases, and the disorder is then referred to as acampomelic campomelic dysplasia, or ACD (Houston *et al*., 1983; Mansour *et al*., 1995; Moog *et al*., 2001). Other features include laryngotracheomalacia (softening of the laryngo-tracheal cartilages) with respiratory deficiencies, ambiguous genitalia, or male to female sex reversal in 75% of XY CD patients, hearing impairment, and a variety of congenital heart defects have been described in a small number of cases (Houston *et al*., 1983).

CD can be identified on prenatal ultrasound examinations based on the shortness and bowing of the long bones (Olney *et al*., 1999), however ACD is often undetectable. Newborns with CD usually die in the first year of life due to respiratory distress. However there are a

few reports of affected infants surviving the neonatal period. In a study published in 2002 (Mansour *et al*., 2002), all five surviving patients analyzed displayed distinctive facial anomalies such as macrocephaly, depressed nasal bridge, hypertelorism (increased distance between the eyes), elongated philtrum and micrognathia with or without cleft palate. The clinical problems of these individuals were primarily of a respiratory nature, including recurrent apnoea sometimes requiring tracheotomy. Later in life, complications included conductive hearing loss and developmental delay (Mansour *et al*., 2002). Because most patients present the symptoms as a result of *de novo* mutations, parents are typically not affected. There are few examples in which both the parent and child are presenting the symptoms, but in all cases the parent is only mildly affected and diagnosed only after the birth of a severely affected child (Leipoldt *et al*., 2007; Mansour *et al*., 2002; Savarirayan *et al*., 2003).

CD is due to haploinsufficiency of the transcription factor *SOX9* on chromosome 17q24.3. *SOX9* was first identified as the candidate gene for CD in patients with reciprocal *de novo* translocations upstream of the *SOX9* coding sequence (Foster *et al*., 1994; Tommerup *et al*., 1993; Wagner *et al*., 1994; Young *et al*., 1992). Subsequently, the characterization of *de novo* heterozygous loss-of-function mutations within the SOX9 coding region were also linked to CD cases (Foster *et al*., 1994; Kwok *et al*., 1995; Meyer *et al*., 1997; Wagner *et al*., 1994). Nonsense, missense, and frameshift mutations in the *SOX9* coding region represent ~90% of CD cases. The remaining cases are the result of *SOX9* deletions, translocations, or inversions upstream of *SOX9*. Nonsense and frameshift mutations of *SOX9* are distributed over the entire coding region, while missense mutations are clustered in the HMG domain, interfering with DNA binding (Meyer *et al*., 1997, McDowall *et al*., 1999) or in the dimerization domain, preventing dimer formation (Bernard *et al*., 2003, Sock *et al*., 2003). Nonsense and most frameshift mutations at the C-terminus result in a SOX9 protein with truncation of the transactivation domain. In mutations at the N-terminus, typically part or the entire HMG domain is missing. The corresponding mutant proteins lacking both the transactivation domain and the HMG domain constitute loss-of-function alleles, while mutant proteins retaining the HMG domain may function as dominant-negative alleles (McDowall *et al*., 1999; Preiss *et al*., 2001).

Transcriptional regulation of *SOX9* involves a ~1 Mb *cis*-regulatory domain upstream of *SOX9*. Translocation and inversion breakpoints that interrupt this upstream sequence are all unique but fall within the same breakpoint clusters: a proximal cluster and a distal cluster located ~400 kb apart (Leipoldt *et al*., 2007). In long-term survivors of CD d*e novo* translocations or inversions with breakpoints upstream of *SOX9* are more likely to be seen than mutations in the *SOX9* coding region, suggesting that they are generally less severe than the intragenic mutations (Leipoldt *et al*., 2007; Pfeifer *et al*., 1999). Recently, lesions have been described that map upstream of the 1 Mb regulatory domain and downstream of *SOX9* in patients with isolated Pierre Robin sequence, a craniofacial disorder characterized by micrognathia, cleft palate and macroglossia (Benko *et al*., 2009; Jakobsen *et al*., 2007). Pierre Robin sequence is also one of the more consistent features of CD and ACD. The identification of mutations on either side of *SOX9* associated with craniofacial defects seen in CD patients indicates that *SOX9* regulatory sequences are covering a much larger genomic region than initially suspected. Further these observations support the existence of tissue specific enhancers acting over a long distance to regulate *SOX9* expression during craniofacial development. The loss or dysregulation of these regulatory regions around *SOX9* may account for the spectrum of craniofacial defects described in CD and ACD. A couple of excellent reviews have recently discussed these findings (Amiel *et al*., 2010; Gordon *et al*., 2009).

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